Declaration by Dr Kyoko Higuchi

1. I am a graduate of the University of Tokyo from where I received my Ph.D. in 1995.

2. As a senior lecturer, I have taught and performed research in applied biology and chemistry in the Tokyo University of Agriculture.

3. I have worked on nicotianamine synthase (NAS) for many years. My publications in this field in the period 1994-2001 are listed in the attached annex.

4. I am named as an inventor for United States Patent Application No. 09/674,337. I have been asked to comment on the difficulties I encountered in purifying the NAS protein and cloning the NAS gene subsequent to performing the work described in Higuchi et al (1994) Plant and Soil 165, 173-9 (Higuchi et al 1994).

5. In Higuchi et al 1994, I reported partial purification of NAS and showed that NAS activity was induced under Fe-deficient condition in barley root. As mentioned in the final paragraph, left hand column, page 178 of Higuchi et al 1994. I then proceeded to attempt to clone NAS based on partial amino acid sequencing of the protein preparation described in Higuchi et al 1994. At this point I should mention that no NAS protein or related protein had previously been cloned. Therefore no sequence information was available to assist in the cloning of NAS.

6. I found that we could not obtain any N-terminal sequence information by subjecting the extract of Higuchi et al 1994 to amino acid sequencing. After many attempts to obtain sequence in this way failed, I attempted cloning of NAS based on an antibody based approach. Once we had obtained an antibody specific to NAS we could use this to purify NAS (for example using the antibody in an affinity column) or to screen

an expression library of clones from a plant that expresses NAS.

7. We generated a polyclonal antibody using the available extract that contained NAS. However, the antibody had only a weak reactivity to NAS protein, because the NAS extract we had used to generate the antibody was not pure enough. We found that the antibody was of no assistance in helping us to clone the NAS gene.

8. It is true that we subsequently did generate a high affinity antibody (as described in Higuchi et al (1999) Soil Sci. Plant Nutr., 45, 681-91. However this antibody was generated using a preparation of NAS obtained by following the procedure described in Higuchi. et al (1999) Plant Physiol., 119, 471-480, and not using the extract of Higuchi et al 1994.

9. I then attempted another approach to clone the NAS gene. We tried to purify NAS using based on its affinity for its substrate. A SAM analogue, S-adenosyl-homocysteine, was fixed to a column through which the NAS extract was passed. However, we found that elutions from the column either contained no protein or contained all of the protein that was present in the initial extract, and therefore affinity purification using the substrate proved unsuccessful.

10. I then attempted to purify NAS using an extract from another species. It had been reported that Basidiobolus Meristoporus, which is one of Zygomycete species, secretes nicotianamine. I tried to perform purification of NAS from Basidiobolus Meristoporus. However, the NAS activity of a crude extract obtained from Basidiobolus Meristoporus was very low. Further, I found that the medium in which Basidiobolus Meristoporus was cultured contained soybean protein and other unfavorable compounds which inhibited enzyme purification.

11. After this attempt, I decided that the best way forward would be to try to develop a

purification procedure which could be used to obtain NAS in a form in which it could be sequenced. As described above purification based on specific affinity purification was unsuccessful, and I was required to develop a procedure that purified NAS but not on the basis of sequence.

12. There are many reasons why protein sequencing may not be successful. Such reasons include low quantities of the protein to be sequenced and the presence of contaminating proteins and chemicals. In the present case a further complication was caused by the fact that the NAS protein is extremely sensitive to degradation. Degradation not only reduces the quantity of NAS but generates degradation products which also interfere with sequencing. In order to prevent degradation of NAS a protease inhibitor needs to be present in the NAS preparation during purification.

13. This means that purifying NAS is difficult because it is not simply a matter of subjecting it to additional purification steps until it is sufficiently pure. Too many purification steps result in loss and degradation of the NAS. Instead the development of a purification procedure requires the finding of a combination of purification steps which results in separation from contaminating proteins and chemicals but does not cause substantial loss or degradation of NAS.

14. After intensive effort on the part of me and my colleagues, and four years after the publication of Higuchi et al 1994, we finally developed a procedure which allowed us to obtain NAS in a form which could be sequenced. As can be seen in the attached table this procedure is much more complicated than the purification procedure used in Higuchi et al 1994, and was definitely not routine to develop. In particular, one reason why the new procedure may be superior to the purification procedure described in Higuchi et al 1994 is that the new procedure is better at removing impurities which have a similar molecular mass to NAS or which cause degradation

of NAS.

15. Upon cloning the gene we deduced that the original NAS extract of Higuchi et al 1994 had in fact consisted of seven isozymes. To some extent this may also have caused difficulties in NAS purification and sequencing. I believe the presence of multiple copies of NAS genes probably contributed to the difficulty in PCR amplification of NAS genes using a primer with sequence deduced from the N terminal amino acid sequence. In order to overcome these difficulties we resorted to using a primer based on EST sequence (as discussed in Example 5 of the present application).

16. Given the difficulties which I encountered in cloning the NAS gene I believe that this gene could not have been cloned by any routine means that was available at the time. As described above, we attempted all available techniques to clone the NAS gene, and none of them worked. It required the development of a special purification technique to obtain NAS protein in a form in which it could be sequenced.

Signed Kyoko Higuchi' Date 06/26/2003

Annex

Publications by Kyoko Higuchi concerning nicotianamine synthase and its function in the period 1994-2001

Higuchi K., Kanazawa K., Nishizawa N K., Chino M., Mori S.
Purification and characterization of nicotianamine synthase from Fe deficient barley roots.
Plant and Soil, 1994, 165 (2) : 173-179.

Higuchi K., Nishizawa N K., Yamaguchi H., Roemheld V., Marschner H., Mori S.
Response of nicotianamine synthase activity to Fe deficiency in tobacco plants as compared with barley.
Journal of Experimental Botany, 1995, 46 (289) : 1061-1063.

Higuchi K., Nishizawa N., Roemheld V., Marschner H., Mori S.
Absence of nicotianamine synthase activity in the tomato mutant 'chloronerva'.
Journal of Plant Nutrition, 1996, 19 (8-9) : 1235-1239.

Higuchi K., Kanazawa K., Nishizawa N K., Mori S.
The role of nicotianamine synthase in response to Fe nutrition status in Gramineae.
Plant and Soil, 1996, 178 (2) : 171-177.

Higuchi K., Suzuki K., Nakanishi H., Yamaguchi H., Nishizawa N. K., Mori S.
Cloning of nicotianamine synthase genes, nevel genes involved in the biosynthesis of phytosiderophores.
Plant Physiology, 1999, 119 (2) : 471-480.

Suzuki K., Higuchi K., Nakanishi H., Nishizawa N. K., Mori S.
Cloning of nicotianamine synthase genes from Arabidopsis thaliana.

Soil Science and Plant Nutrition, 1999, 45 (4) : 993-1002.

Higuchi K., Nakanishi H., Suzuki K., Nishizawa N. K. Mori S.
Presence of nicotianamine synthase isozymes and their homologues in the root of graminaceous plants.
Soil Science and Plant Nutrition, 1999, 45 (3) : 681-691.

Higuchi K., Watanabe S., Takahashi M., Kawasaki S., Nakanishi H., Nishizawa N. K., Mori S.
Nicotianamine synthase gene expression differs in barley and rice under Fe-deficient conditions.
Plant Journal, 2001, 25: 159-168.

Higuchi K., Tani M., Nakanishi H., Yoshiwara T., Goto F., Nishizawa N. K., Mori S.
The expression of a barley HvNAS1 nicotianamine synthase gene promoter-gus fusion gene in transgenic tobacco is induced by Fe-deficiency in roots.
Biosci. Biotechnol. Biochem., 2001, 65 (7) : 1692-1696.

Higuchi K., Takahashi M., Nakanishi H., Kawasaki S., Nishizawa N. K., Mori S.
Analysis of transgenic rice containing barley nicotianamine synthase gene.
Soil Science and Plant Nutrition, 2001, 47: 315-322.

TABLE COMPARING PURIFICATION METHODS

Higuchi et al (1994)
Plant and Soil
165, 173-179

TSK gel Butyl-Toyopearl 650M (Fractogel TSK Butyl-650M, Merck) coloumn (1 x 11cm) equilibrated with starting buffer for hydrophobic chromatography synthase. NAS eluted with water containing 1% glycerol.

↓

The enzyme fraction was equilibrated with the starting buffer (1 mM K-P, 0.1 M KC1, pH 8.0) for the hydroxylapatite chromato graphy, concentrated, and applied to a hydroxlapatite 100~ 350 mesh (Nacalai Tesque Japan) column (1.5 x 2.7cm) equilibrated with the starting buffer. NASwas eluted with buffer A (4 mM K-P, 0.1 M KCI, pH 8.0 with KOH), and buffer B (8 mM K-P, 0.1 M KCI, pH 8.0).

↓

SDS-PAGE was carried out at room temperature using 12.5% acrylamide slab gels. After SDS PAGE, the gel was stained with 0.3 M $CuCl_2$ and was horizontally cut to several fractions nearby 30 Kda. Each fragment was destained with 0.25 M EDTA/0.25 M Tris(pH 9.0)

↓

Polypeptides were eluted by electroelution with the buffer containing 7 mM Tris, 35 mM glycine and 1% (v/v) glycerol.

(From Higuchi et al.,1994, Right hand column, page 174).

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09/674,337

TSK gel Butyl Toyopearl 650M column (10 ml bed volume per 100g of roots), equilibrated with the adsorption buffer and eluted with elution buffer [10 mM Tris, 1mM ETDA, 3 mM DTT, 0.1 mM p-APMSF, 5% glyceroland 0.05% 3-[(3-chloramidopropyl) dimethylammonio] propanesulfonic acid (hereinafter called CHAPS), pH 8.0].

↓

A hydroyapatite 100-350 mesh (Nacalai Tesque), equilibrated with the adsorption buffer (1mM KP, 10 mM KC1, 3 mM DTT and 0.1 mM p-APMSF, pH 8.0), was prepared at 10 ml per protein 100 mg and the fractions containing nicotianamine synthase were loaded. Nicotianamine synthase was passed through without adsorption.

↓

The passed through fraction was loaded onto TSK gel Butyl Toyopearl 650M column (1 ml bed volume per 10 mg of protein), and nicotianamine synthase was eluted as described above for the first step.

↓

The active fraction was loaded onto a DEAESepharose FF column (5 ml bed volume per 25 mg of protein, Pharmacia) equilibrated with the adsorption buffer (20 mM Tris, 1 mM EDTA, 3 mM DTT, 0.1 mM p-APMSF and 0.05% CHAPS, pH 8.0) and eluted with stepwise gradient elution of potassium chloride concentration of 0.05 M, 0.1 M, 0.15 M and 0.2 M. Nictianamine synthase was eluted at 0.15 M of KC1 concentration.

↓

The active fraction was loaded onto the Ether Toyopearl 650M column (10 ml bed volume per 100g of roots), equilibrated with adsorption buffer [20 mM Tris, 1 mM EDTA, 3 mM DTT, 1.2 M $(NH_4)_2SO_4$ and 0.1 mM p-APMSF, pH 8.0]. Nicotianamine synthesis was not absorbed and passed through the column

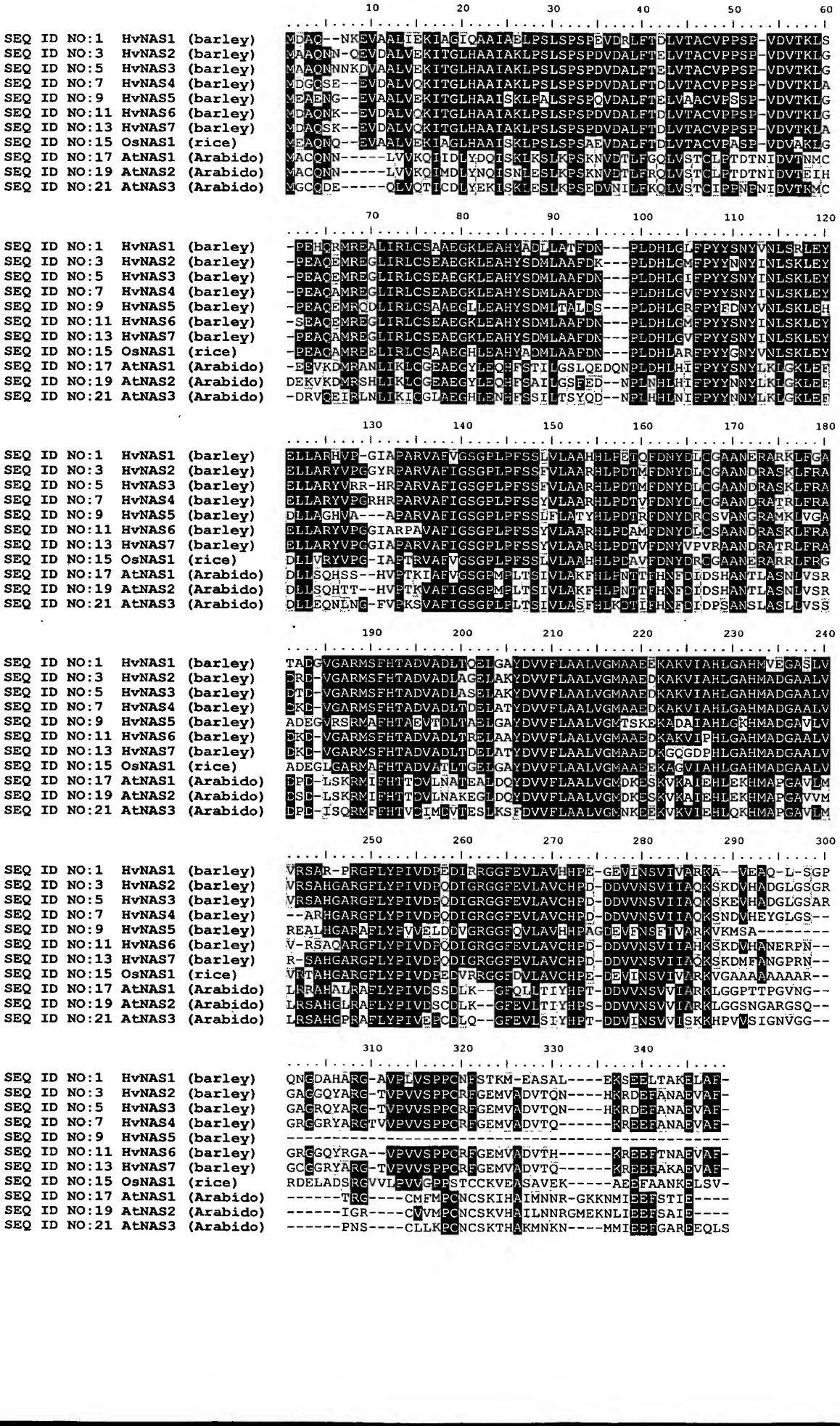
↓

The passed through fraction was loaded onto TSK gel Butyl Toyopearl 650M column and fractions containing nicotianamine synthase were eluted.

↓

SDS-PAGE using 11% acrylamide slab gels. After SDS-PAGE the gel was stained with 0.3 M copper chloride and the separated bands were cut out. The gel fragments were destained with 0.25 M EDTA/0.25 M Tris (pH 9.0) and homogenized with the extraction buffer (1% SDS, 25 mM Tris and 192 mM glycine). Each homogenate was electroeluted with SDS-free buffer (25 mM Tris and 192 mM glycine) and peptide was recovered.

(From Examples 3 of United States Patent Application No. 09/674,337)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SEQ ID NO:3 HvNAS2 (barley)
SEQ ID NO:5 HvNAS3 (barley)
SEQ ID NO:7 HvNAS4 (barley)
SEQ ID NO:9 HvNAS5 (barley)
SEQ ID NO:11 HvNAS6 (barley)
SEQ ID NO:13 HvNAS7 (barley)
SEQ ID NO:15 OsNAS1 (rice)
SEQ ID NO:17 AtNAS1 (Arabido)
SEQ ID NO:19 AtNAS2 (Arabido)
SEQ ID NO:21 AtNAS3 (Arabido)